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Aromatic *N*-Deacylation by Chick-Kidney Mitochondria

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During an investigation of the metabolism of some *para*-substituted anilines it became desirable to convert the excreted *N*-acetyl compounds into their arylamines by enzymic rather than by acid hydrolysis, so reducing the risk of altering other parts of the metabolite molecules. Kynurenine formamidase of mammalian liver (Mehler & Knox, 1950) and of *Neurospora* (Jakoby, 1954), the only enzymes of this type described, appeared not to be suitable because of their high specificity for *o*-formamido compounds. There was, however, evidence that an enzyme more appropriate to our requirements was to be found in chick kidney. Shaffer & Bieter (1950) reported that after giving sulphanilamides to chicks much less of the N^4-acetyl conjugates could be found in kidney than in other tissues or blood. They also found that administered N^4-acetylsulphanilamides were excreted in the urine largely as the free bases; this was in contrast with mammalian species. Finally, these workers showed that chick-kidney homogenates deacetylated acetylsulphanilamides at a rapid rate.

Some of the properties of the chick-kidney enzyme have been explored and are now reported. In spite of the superficial similarity of the reaction it catalyses, the enzyme is quite distinct from kynurenine formamidase.

EXPERIMENTAL

Materials

Substrates. A few of the compounds tested as substrates were of commercial origin. In most cases the arylamines, obtained commercially, were acylated with the appropriate acid anhydride or acid chloride. Formylation was carried out with acetic formic anhydride (Dalgliesh, 1952). In every case the compound was recrystallized from a suitable solvent (e.g. aqueous ethanol) until all detectable arylamine had been removed. The *p*-acetamido derivatives of 3-phenoxypropionic acid, 4-phenoxybutyric acid and 5-phenoxyvaleric acid were kindly provided by Dr A. G. Caldwell. Compounds in the benzamido series were generously prepared for the present purpose by Dr J. H. Gorvin.

Buffers. Buffered solutions at a concentration of 0·1 or 0·2 M were prepared in glass-distilled water. 2-Amino-2-hydroxymethylpropane-1:3-diol (tris) and 2-amino-2-methylpropane-1:3-diol (ammediol) were obtained from L. Light and Co. Ltd. The pH values of all solutions were measured at the concentration at which they were used, with a glass electrode assembly (W. G. Pye and Co. Ltd.) and temperature corrections were applied where necessary.

Other reagents. Most reagents were of A.R. grade and were used as such; others were purified, when necessary, by some standard procedure appropriate to the particular compound. *p*-Chloromercuribenzoic acid was a gift from Dr L. A. Stocken; 2:3-dimercaptopropanol was bought from Boots Pure Drug Co. Ltd. and was not purified. Tween 20 (Honeywill-Atlas Ltd.) and Triton X-100 (Rohm

and Haas Co., Philadelphia, Pa., U.S.A., and bought through Charles Lenning and Co. Ltd., London) were also used as received.

Enzyme preparations

Cross-bred fowls of both sexes and of different ages were killed by air embolism or by cervical section. Kidneys were quickly removed and either used directly (after chilling) or stored at −15°. Activity remained reasonably high during storage; over a period of 3 months there was a loss of only 15%.

Fresh or thawed tissue was homogenized in about 10 vol. of chilled medium in a loose-fitting, all-glass homogenizer of the type described by Potter & Elvehjem (1936) and held in an ice bath. The medium used was 0·25 M-sucrose containing mM-tris and mM-disodium ethylenediaminetetra-acetate (EDTA); it had a pH of 7·1. After centrifuging for 10 min. at 700 *g* the supernatant was centrifuged for a further 10 min. at 15 000 *g* in the no. 69402 angle head (previously chilled to −3°) of the MSE Magnum centrifuge with high-speed attachment (Measuring and Scientific Equipment Ltd.). After removal as completely as possible of the supernatant together with a shallow layer of loosely packed particles, the residue was transferred to the homogenizer and resuspended in the same volume of the sucrose–tris–EDTA medium as had been used originally. For experiments on the effects of metal ions EDTA was omitted throughout. Such suspensions, particularly in the presence of EDTA, were fairly stable at −15° but were not usually kept for more than 5 days. Suspensions prepared in buffered-electrolyte solutions lost activity much more rapidly on storage.

Rat liver was used as a source of kynurenine formamidase. Adult animals of the Laboratory Wistar strain were killed by cervical section; the livers were removed at once, chilled and homogenized in 5–10 vol. of 0·05 M-phosphate buffer, pH 7·4. Homogenates were centrifuged as described above and the supernatant from the second centrifuging was used in most experiments without further treatment. Such preparations were very stable at −15°. For comparative experiments on the effects of metal ions enzyme partially purified by the method outlined by Mehler & Knox (1950) was used.

Measurement of activity

Conditions were varied to suit experimental requirements. Typically, reactions were carried out in 4 in. × ½ in. test tubes held in a water bath at 38° and containing 1 μmole of substrate in 0·5 ml. of 0·1 or 0·2 M-phosphate buffer, pH 7·2–7·3, and were started by the addition of 0·5 ml. of chick-kidney suspension diluted in the sucrose–tris–EDTA medium, or of rat-liver preparation diluted in water. After 60 min. the reaction was stopped by the addition of either 4 ml. of 0·37 N-perchloric acid or of 2 ml. of 0·11 M-zinc acetate followed by 2 ml. of 0·09 N-NaOH; the contents of the tubes were thoroughly mixed and precipitates removed by centrifuging. The amount of liberated arylamine was determined in the supernatant solutions. Results were corrected for a small amount of non-enzymic hydrolysis that occurred with the more labile substances. A small correction for tissue blank had also to be applied when determinations were made in u.v. light.

Analytical methods

Arylamines. Products of enzymic deacylation were measured by several different spectrophotometric methods. A Unicam model SP. 500 instrument with a light path of 1 cm. was used throughout. (*a*) To amine in 2 ml. of a perchloric acid supernatant were added successively 1 ml. of 2 N-HCl, 0·5 ml. of 0·1% $NaNO_2$ solution and 0·5 ml. of 0·5% ammonium sulphamate solution, with intervals of at least 5 min. between the second and third additions. The diazonium compound was coupled with 1-naphthol by adding 1 ml. of a freshly prepared solution containing 1 vol. of 12% (w/v) 1-naphthol in ethanol and 10 vol. of 3 N-NaOH. Intensity of the colour, which developed immediately and was stable for at least 2 hr., was measured at 490 mμ. This method was suitable for determination of *ortho*- and *para*-substituted anilines (except *o*-aminophenol, *p*-phenylenediamine and *p*-dimethylaminoaniline) of the naphthylamines and of 4-aminoantipyrin. (*b*) For the determination of *meta*-substituted anilines diazotization was performed as in (*a*) and colour developed by adding 1 ml. of an aqueous 0·3% solution of *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bratton & Marshall, 1939); intensity was measured at 550 mμ. (*c*) A method of wide applicability was to form the Schiff base of the arylamine with *p*-dimethylaminobenzaldehyde at pH 2·0–2·2 (Venkataraman, Venkataraman & Lewis, 1948). A sample of the supernatant after treatment with zinc acetate and NaOH was brought to 4·5 ml. with water and treated with 0·5 ml. of 0·5% solution of the reagent in acetic acid. Extinctions were measured at 445 mμ after about 30 min.; they remained constant for some hours. In later experiments it was found possible to increase sensitivity twofold and to save much time by adding 3 ml. of a modified reagent directly to the 1 ml. of reaction mixture. The modified reagent was made freshly each day by dissolving 240 mg. of *p*-dimethylaminobenzaldehyde in 12 ml. of acetic acid and diluting with 30 ml. of redistilled ethanol and 50 ml. of water. Even relatively large amounts of suspension, containing up to 100 μg. of protein, did not interfere with colour production and gave by themselves extinctions less than 0·02. (*d*) For the measurement of deacylation of two of the substrates none of the above methods was suitable, and in these instances determinations were based upon differences in u.v. absorption of acid solutions. Samples of the perchloric acid supernatants were suitably diluted in 0·1 N-HCl and readings were made at 240 mμ for the conversion of *p*-aminoacetanilide (ε 13 400) into phenylenediamine (ε 210) and at 242 mμ for the conversion of *p*-dimethylaminoacetanilide (ε 15 100) into *p*-dimethylaminoaniline (ε 1120). In a few of the experiments with this substrate the deacylation of *p*-acetamidoanisole (*p*-acetanaside; ε 11 000) to *p*-anisidine (ε 150) was determined in the same way at 245 mμ.

For each method separate calibrations were made with purified samples of the arylamines concerned. With several substrates it was possible to make comparisons of the different methods and these were in excellent agreement.

Protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used for determination of protein and was standardized with crystalline bovine serum albumin (The Armour Laboratories, Eastbourne). With samples of the particulate suspension it was found

advisable for quantitative recovery of protein to allow treatment with the alkaline copper reagent to continue for 2 hr.

RESULTS

Intracellular localization of activity

Homogenates of chick kidney, after centrifuging at 700 *g*, regularly showed a high order of activity ranging from 170 to 320 μmoles of *p*-acetamidoanisole hydrolysed/g. of tissue/hr. When such suspensions were centrifuged at 15 000 *g* only traces of activity could be measured in the supernatant; at least 80 % of the original activity, however, could be recovered in the resuspended residue. A second sedimentation and resuspension of the particles was accompanied by a slight decrease in total activity, but again only about 1 % of this could be found in the supernatant. Essentially the same results were obtained, though with a rather greater loss of total activity, when the particles were suspended in various concentrations of phosphate buffer at pH 7·3.

Several treatments were used in unsuccessful attempts to release the enzyme from its particulate association. After each treatment the suspension was centrifuged at 15 000 *g* and activity sought in the supernatant; recovery of total activity was determined either with a sample taken before centrifuging or from activity of the sedimented residue after resuspension in the sucrose medium. Although, as has been found by other workers with liver mitochondria, surface-active agents brought about a release of large amounts of protein, there was no associated release of activity. An outline of the results of these experiments is given in Table 1. Repeated freezing and thawing was also unsuccessful. In all further work suspensions of the sedimented particles were used.

Table 1. *Effect of some surface-active agents on protein distribution and total deacylating activity*

Portions of chick-kidney particulate suspension (5–6 mg. of protein/ml.) were treated at 5° and pH 7·2 for 20 min. Activity values are given as percentages of those found in untreated suspensions; 'protein released' is the percentage of total protein found in the 15 000 *g* supernatant after treatment. Activities of the supernatants, which never exceeded 2–3 % of the total, are not given.

Treatment	Protein released (%)	Total activity (%)
Control suspension	9	100
Cetyltrimethylammonium bromide (0·1 %)	31	3
Tween 20 (0·1 %)	51	105
Sodium deoxycholate (0·12 %)	60	73
Triton X-100 (0·12 %)	70	96
Digitonin (1 %)	94	13

Effect of time and enzyme concentration on rate of reaction

With a suitable amount of enzyme the deacylation of mM substrate was linear with time up to about 50 % of completion (Fig. 1). During a fixed incubation period a similar proportionality between deacylation and enzyme concentration (Fig. 2) was found for all substrates tested in this respect. For

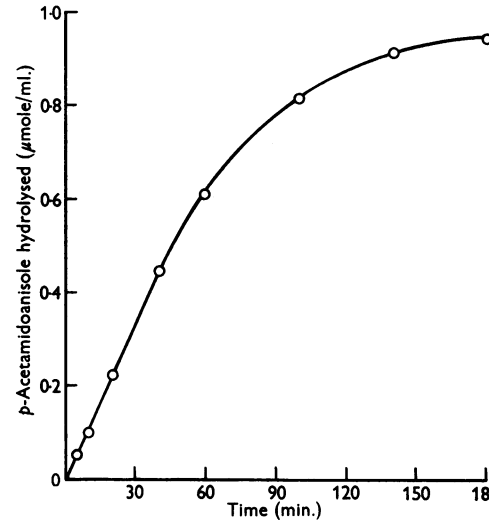

Fig. 1. Effect of time on course of reaction. *p*-Acetamidoanisole (10 μmoles) in 10 ml. of 0·1 M-phosphate buffer, pH 7·2, was incubated with enzyme (230 μg. of protein) at 38°. Enzyme was added after temperature equilibration and then samples (0·5 ml.) were removed at intervals into perchloric acid and analysed for *p*-anisidine.

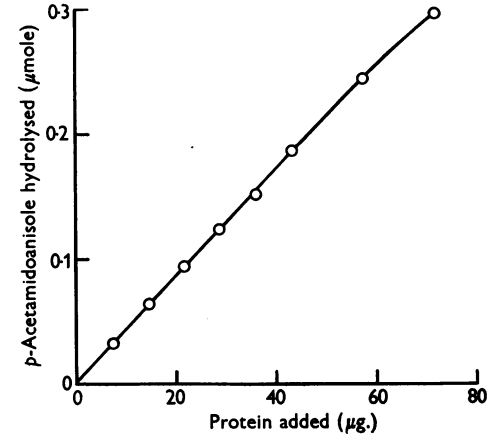

Fig. 2. Effect of varying enzyme concentration. Increasing amounts of chick-kidney suspension were incubated for 60 min. at 38° with 1 μmole of *p*-acetamidoanisole in 1 ml. of 0·1 M-phosphate buffer, pH 7·2.

the accurate determination of rates the enzyme concentration was adjusted so as to allow not more than 20% of complete hydrolysis/hr.

Influence of pH *and of different buffers*

In 0·05M-phosphate buffer a well-defined peak of activity was found at pH 7·2–7·3; the curve relating activity to pH was continued into the more alkaline range by 0·05M-$NaHCO_3$–Na_2CO_3 buffer (Fig. 3). With no other buffer system could a clear optimum of activity be established, although in glycine buffer (Fig. 3) there was indication of an approaching peak towards the acid end of the useful range of this buffer. Pyrophosphate buffer produced a broad range of high activity extending from pH 7·6 to 8·4, with a slow decline on either side. In the presence of tris–HCl, tris–acetic acid, ammediol–HCl and triethanolamine–HCl buffers activity rose sharply from the acid end of the ranges to reach a plateau of activity between pH 8·2 and 9·2. On these plateaux rates were much the same in each of the four buffers and approximately equal to activity at the optimum found in phosphate buffer. Diethylbarbiturate (Fig. 3) and borate buffers were inhibitory; in each, activity was greatest at the highest pH tested (pH 9·1 and 9·8 respectively) and was not more than 40% of that found in phosphate buffer at pH 7·3.

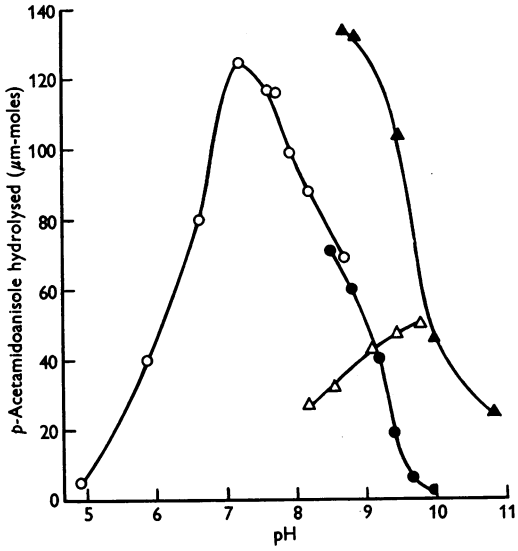

Fig. 3. Effect of pH and of buffer ion. *p*-Acetamidoanisole (1 μmole) was incubated with suspension (9 μg. of protein) in 1 ml. of 0·05M-KH_2PO_4–Na_2HPO_4 (○), $NaHCO_3$–Na_2CO_3 (●), glycine–NaOH (▲) or sodium diethylbarbiturate–HCl (△) buffer for 60 min. at 38°.

Influence of temperature

The initial rate of deacylation of *p*-acetamidoanisole in 0·1M-phosphate buffer, pH 7·2, was measured during 15 min. at temperatures between 14° and 50°. An optimum temperature of near 44° was found for this incubation period (Fig. 4). From an Arrhenius plot of the rates in the range 19–40° the energy of activation was calculated to be 15 300 cal. Above 40° heat inactivation of the enzyme was increasingly apparent.

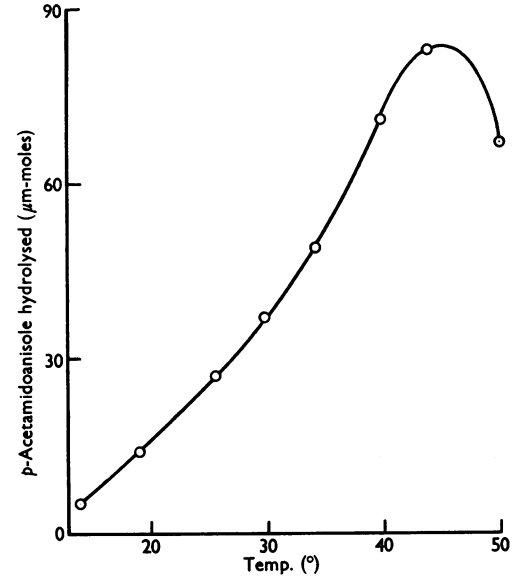

Fig. 4. Influence of temperature on the deacetylation of *p*-acetamidoanisole. Tubes containing 1 μmole of substrate in 1 ml. of 0·1M-phosphate buffer, pH 7·3, were incubated for 7 min. at the indicated temperatures. The reaction was started by adding 0·05 ml. of suspension and stopped after 15 min. with 4 ml. of perchloric acid.

Inhibitors

A variety of compounds was tested for possible effects on the rate of deacylation of *p*-acetamidoanisole. Acetate (adjusted to pH 7·3) was without effect at 10 mM and inhibited only to the extent of 18% at 100 mM. Monochloroacetate caused a 12% reduction in rate at 5 mM and a 62% reduction at 50 mM. Monoiodoacetate was rather more toxic; it reduced the rate by 17% at 2 mM and by 50% at 10 mM.

The enzyme (prepared in the absence of EDTA) was sensitive to inhibition by *p*-chloromercuribenzoate (Fig. 5); at 10 μM this compound caused about 30% inhibition and at 100 μM complete inhibition. Three compounds containing thiol groups were tested for their ability to reverse this inhibition, but in every condition tested only partial reactivation could be demonstrated. Portions of

chick-kidney suspension, with and without inhibitor at 50 μM, were incubated in 0·25 M-sucrose containing 0·05 M-phosphate buffer, pH 7·2, at 38°. After 15 min. samples were diluted and added to tubes containing buffered substrate and different concentrations of the thiol compounds; the mean rate of hydrolysis during an incubation period of 1 hr. was then determined. In these conditions samples pre-incubated in the presence of inhibitor had 40–50 % of the activity of portions pre-incubated in its absence. Reduced glutathione at 2·5 mM consistently reversed inhibition to the greatest extent observed, but activity, as determined during the fixed time interval employed, never reached more than 80 % of the control level. Cysteine at the same or at higher concentrations was slightly less effective than glutathione. At the highest concentrations tested (25 mM) both compounds caused a slight, variable (5–15 %) stimulation of activity in control samples. At no concentration tested (0·25–25·0 mM) did 2:3-dimercaptopropanol effect any reversal of the inhibition produced by *p*-chloromercuribenzoate; at the higher concentrations it was itself slightly inhibitory.

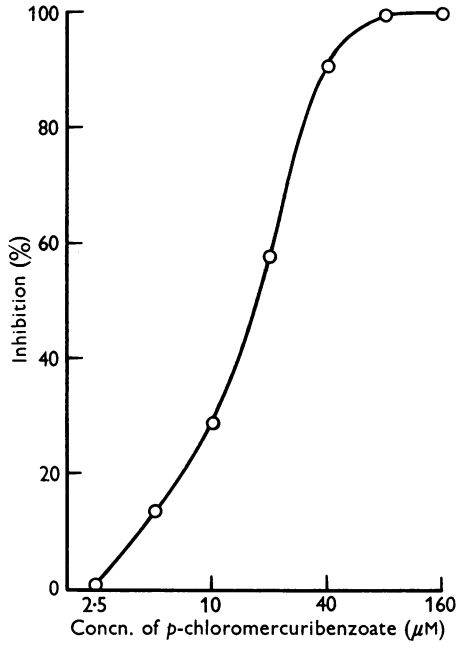

Fig. 5. Inhibition by *p*-chloromercuribenzoate of the deacetylation of *p*-acetamidoanisole. Substrate (1 μmole) and chick-kidney suspension prepared without EDTA (12 μg. of protein) were incubated with the indicated concentrations of inhibitor at 38° in 1 ml. of 0·1 M-phosphate buffer, pH 7·2.

Table 2. *Inhibition by metals of the deacetylation of p-acetamidoanisole by suspensions of chick-kidney particles*

Incubations were for 60 min. in 1 ml. of tris–acetic acid buffer containing 1 μmole of substrate and the indicated concentrations of inhibitor. Results are the mean values of at least two experiments; control tubes without inhibitor were included in each series. Enzyme was prepared without EDTA.

	Inhibition (%)			
Compound	Concn. 0·2 μM	Concn. 2·0 μM	Concn. 20·0 μM	Concn. 200·0 μM
$HgCl_2$	22	76	100	—
$CuCl_2$	9	78	95	—
$CdCl_2$	9	76	98	—
Zinc acetate	10	66	100	—
$AgNO_3$	0	42	100	—
$Pb(NO_3)_2$	—	14	69	83
$Ni(NO_3)_2$	—	11	—	80

From Table 2 it can be seen that the enzyme was very sensitive to inhibition by metal ions. In the presence of 2 μM-Hg^{2+}, -Cu^{2+}, -Cd^{2+} or -Zn^{2+} ions rates of deacylation were reduced to 20–35 % of control values. Even at 0·2 μM these metals caused distinct inhibition, most marked for Hg^{2+} ions. A slightly less-pronounced effect was produced by Ag^{+} ions and Pb^{2+} and Ni^{2+} ions were roughly one-tenth as inhibitory.

Arsenite produced a 62 % inhibition at 2 mM; at the same concentration cyanide was without effect.

Particle suspensions prepared on separate occasions in the absence of EDTA exhibited variable activities when these were determined also in the absence of EDTA. On the other hand, when the chelating agent was added (at 0·5 mM) to the assay tubes rates in the less-active preparations were usually increased; the largest stimulation seen in these conditions was approximately twofold.

Substrate specificity

Ability of the chick-kidney particles to deacylate a range of monosubstituted acylanilides was explored. Initial reaction rates were measured as already described; enzyme concentrations were necessarily varied over a wide range but, as before, values were calculated from amounts which resulted in the release of less than 0·2 μmole of arylamine from 1 μmole of substrate/hr. Within each experiment measurements of the rate of deacylation of a reference substrate (usually *p*-acetamidoanisole) were always included. Determinations of relative rate made in this way with different suspensions and at different times agreed well.

With several substrates initial rates were measured over a wide range of substrate concentration. Apparent K_m values were calculated from double reciprocal plots of the data (Lineweaver & Burk, 1934) fitted by the method of least squares.

Effects of ring substitution. The effect of introducing a *para* substituent into acetanilide was

generally to increase the relative rate of deacylation (Table 3). Only two substrates in this series, *p*-acetamidoaniline and *p*-acetamidoarsonic acid, were caused to react at a substantially slower rate. Determination of the apparent K_m values of some such substrates (Table 4) indicated that the increased rate at 1 mM concentration after *para* substitution was due at least in part to an increased affinity for the enzyme. From these values it can be seen that the relative rates of deacylation of other *para*-substituted compounds at 1 mM concentration is a rough indication of their affinities.

Ortho substitution had an opposite effect and was invariably followed by a profound fall in the rate of deacylation; *p*-acetamidobenzoic acid, for instance, was deacylated about 1000 times as rapidly at mM concentration as was the *ortho* compound. Rates of deacylation of these compounds (Table 5) were sometimes so slow as to prevent a determination of apparent K_m values. For *o*-acetamidoanisole the apparent K_m was found to be 10·2 mM (max. rate 0·72 μmole/mg. of protein/hr.); for *o*-acetamidochlorobenzene it was 3·4 mM (max. rate 1·3 μmoles/mg. of protein/hr.).

Substitution in the *meta* position had a tendency to reduce the rate of reaction (Table 5), but its effects were very much weaker than those of the corresponding *ortho* substitution. Only one substituent in this position, chlorine, effectively increased the rate over that of the unsubstituted compound.

Influence of the N-*acyl group*. With the six substrates tested in this respect the rates of deacylation of chloroacetyl derivatives were much faster than those of the corresponding acetyl compounds (Table 3). With acetanilide, for instance, the rate of deacylation was increased about eightfold by the introduction of chlorine. In the chloroacetyl series, however, the effect of *para* substitution was much less marked than in the acetyl series (Table 3); in fact the *p*-chloroacetyl derivatives of compounds containing a carboxylic group were split at about half the rate of chloroacetanilide. The apparent K_m values of four of these compounds were determined and are given in Table 4.

A similar large increase in the relative rate of deacylation of *ortho*- and *meta*-substituted compounds after *N*-chloroacetylation was also observed. For example *o*-chloroacetamidobenzoic acid was deacylated about 25 times as rapidly as *o*-acetamidobenzoic acid; it had an apparent K_m of 0·43 mM but its maximal rate of reaction was still only 2·1 μmoles/mg. of protein/hr.

Upon *N*-dichloroacetylation the relative rates fell to values very little higher than those of the *N*-acetyl derivatives.

Formylation was accompanied in every case tested by a marked fall in the relative reaction rate. With formanilide the relative rate was decreased to 8% and with *p*-formamidoanisole to 5% of

Table 3. *Influence of* para *substitution on the rate of deacylation of acetanilide and chloroacetanilide*

Substrates (1 mM) were incubated with enzyme in 0·1 M-phosphate buffer, pH 7·3, for 60 min. at 38°. Enzyme concentrations were adjusted to allow not more than 20% of substrate to be changed. Liberated arylamines were measured by methods described in the Experimental section. Each value is the mean of two or more determinations. Rates given are all relative to that of acetanilide.

Substituent	Relative rate of deacylation	
	N-Acetyl	*N*-Chloroacetyl
$AsO(OH)_2$	10	—
NH_2	60	—
CN	90	—
H	100	840
OH	115	—
$CO{\cdot}CH_3$	145	1300
$CO{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$	150	925
NO_2	170	—
CHO	180	—
$O{\cdot}CH_2{\cdot}CH_2OH$	200	—
$O{\cdot}CH_3$	220	2530
CO_2H	220	500
$SO_2{\cdot}NH_2$	220	—
$N(CH_3)_2$	260	—
$O{\cdot}[CH_2]_{2-4}{\cdot}CO_2H$	280	—
$O{\cdot}CH_2{\cdot}CH_3$	310	—
CH_3	330	—
$CH_2{\cdot}CO_2H$	330	515
Cl*	380	—

* Compared, because of its low solubility, at a concentration of 0·5 mM.

Table 4. *Apparent* K_m *values of some* para*-substituted acylanilides*

Determinations were made as outlined in Table 3 with substrate concentration systematically varied in the range 0·05–5·0 mM. Maximal rates are given as μmoles/mg. of protein/hr.; K_m values are mM concentrations.

Substituent	*N*-Formyl		*N*-Acetyl		*N*-Chloroacetyl	
	K_m	Max. rate	K_m	Max. rate	K_m	Max. rate
H	7·4	5·6	0·91	10	0·14	32
$O{\cdot}CH_3$	1·2	16	0·59	18	0·61	122
CO_2H	1·3	17	0·23	5·4	1·0	15
$CH_2{\cdot}CO_2H$	2·6	19	0·078	18	0·12	38

those found with the acetamido compounds; with *p*-aminobenzoic acid, *p*-aminophenylacetic acid and *p*-aminohippuric acid the difference was less marked and the formyl derivatives were deacylated at 75, 20 and 40% respectively of the rate of the corresponding acetyl compound. From Table 4 it can be seen that the slower deacylation of formamido compounds at mM concentration is probably a reflexion of the relatively high apparent K_m values of these compounds.

The effect of increasing the length of the *N*-acyl chain was also investigated. Deacylation, on the basis of relative rate, was most rapid with the *p*-propionamido derivatives of benzene, anisole and phenetole, and with the *p*-acetamido derivatives of benzoic acid, phenylacetic acid and hippuric acid. The relationship between relative rate and chain length is exemplified for these two groups in Fig. 6.

Table 5. *Influence of ring substitution on the rate of deacetylation of acetanilide*

Measurements of relative rate were made as outlined in Table 3. The rate of deacetylation of acetanilide is given a value of 100.

Substituent	Relative rate		
	ortho	*meta*	*para*
OH	0·3	38	115
NO_2	25	62	170
$O{\cdot}CH_3$	1·6	—	220
CO_2H	0·2	35	220
CH_3	<0·1	80	330
Cl	15	160	380

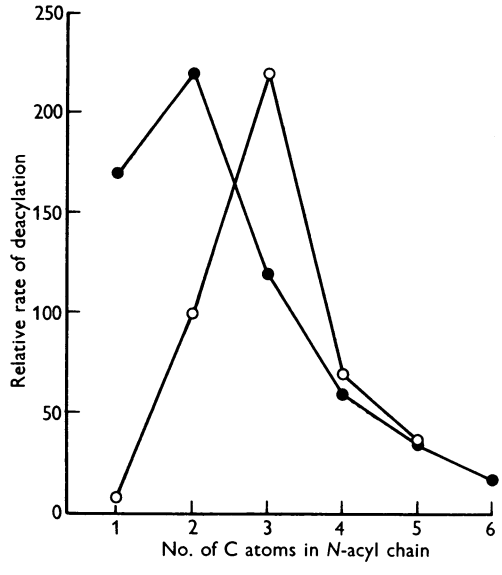

Fig. 6. Effect of *N*-acyl chain length on relative rate of hydrolysis. In the series $R{\cdot}C_6H_4{\cdot}NH{\cdot}CO{\cdot}[CH_2]_n{\cdot}H$ 1 μmole of substrate was incubated with suitably diluted chick-kidney suspension in a total volume of 1 ml. of 0·1 M-phosphate buffer, pH 7·3, for 60 min. at 38°. The substituents R were H (○) and *p*-CO_2H (●).

In Table 6 are listed the apparent K_m values of some longer-chain derivatives of *p*-aminobenzoic acid and *p*-aminophenylacetic acid. With both compounds there was an unexpected fall in apparent K_m in passing from the *N*-butyryl to the *N*-valeryl derivative; determinations made on separate occasions confirmed this finding. On further increasing the length of the *N*-acyl chain on *p*-aminobenzoic acid (Table 6) the apparent K_m value increased only slightly.

N-Succinyl derivatives of several of the compounds already mentioned were tested, but none was deacylated at a measurable rate. The effect of branched chains was not investigated.

N-Benzoyl derivatives. Low solubilities of benzamido compounds limited the investigation of substrates of this type, and only derivatives of *p*-aminobenzoic acid were tested. *p*-Benzamidobenzoic acid was hydrolysed about one-fifth as fast as acetanilide or one-tenth as fast as *p*-acetamidobenzoic acid at the same concentration. Its apparent K_m was found to be 1·6 mM and it reacted maximally at a rate of 3·7 μmoles/mg. of protein/hr. (cf. Tables 4 and 6). Introduction of a nitro group into the *para* position of the benzamido moiety was without effect on the relative rate; the same substituent in the *meta* position decreased the rate slightly, but in the *ortho* position it reduced the rate by a factor of 50.

Miscellaneous substrates. Other than derivatives of aniline very few compounds were tested as substrates. 2-Acetamidonaphthalene was deacylated about four times as rapidly as acetanilide; the 1-isomer was apparently not attacked. A relatively slow deacylation of 4-acetamidoantipyrin (about 30% of the rate of acetanilide) was also demonstrated.

Table 6. *Effect of* N*-acyl chain length on* K_m *values of derivatives of* p*-aminobenzoic acid and* p*-aminophenylacetic acid*

Values were determined as described in Table 4, in which are given values for substrates with shorter chains. Substrates had the structure $R{\cdot}C_6H_4{\cdot}NH{\cdot}CO{\cdot}[CH_2]_n{\cdot}H$.

R	*n*	K_m	Max. rate
CO_2H	2	1·7	6·4
CO_2H	3	1·3	2·0
CO_2H	4	0·47	1·0
CO_2H	5	0·54	1·5
$CH_2{\cdot}CO_2H$	2	0·70	30
$CH_2{\cdot}CO_2H$	3	0·76	6·8
$CH_2{\cdot}CO_2H$	4	0·15	2·2

Experiments with rat-liver enzyme

Some of the properties of kynurenine formamidase have been described by Mehler & Knox (1950) and by Jakoby (1954). So as to provide some further points of comparison with the chick-kidney enzyme a few more experiments were done. *o*-Formamidobenzoic acid (formylanthranilic acid) was used as a reference substrate.

Inhibitors. Partially purified enzyme was not inhibited by *p*-chloromercuribenzoate at 0·5 mM, the highest concentration tested. At 2 mM-$HgCl_2$, zinc acetate and $CuCl_2$ caused inhibitions of 47, 45 and 9% respectively. It may be recalled (Table 2) that at 2 μM these compounds caused a greater inhibition of the chick-kidney enzyme.

Influence of ring substitution and N*-acyl group.* Formanilide was deacylated at a relatively slow rate; the relative effectiveness of various *ortho* substituents in increasing rate was confirmed and the *o*-chloro derivative was included. The decreasing order of effectiveness was found to be CO_2H, NO_2, Cl, H, CH_3, *o*-methylation resulting in a decrease in rate as compared with formanilide.

It was also confirmed that *para*-substituted compounds were deacylated at a very slow rate; *p*-acetamidobenzoic acid, for example, was hydrolysed at less than 0·02% of the rate of formylanthranilic acid.

As reported by Mehler & Knox (1950), *o*-formamido compounds were hydrolysed considerably faster than the *o*-acetamido derivatives. In the present investigation it was established that the rate increased again with propionylanthranilic acid (Table 7) but thereafter declined with increasing chain length. Chloroacetylation produced a substrate which was deacylated almost as fast as the relative rate reported for formylkynurenine. *o*-Benzamidobenzoic acid was hydrolysed at a very slow, but still measurable, rate.

Table 7. *Effect of different* N*-acyl groups on the hydrolysis of anthranilic acid derivatives by rat-liver enzyme*

Relative rates of reaction were measured as described in Table 3, except that a suitably diluted rat-liver preparation was used.

N-Acyl group	Relative rate
Formyl	100
Acetyl	9
Chloroacetyl	375
Propionyl	27
Butyryl	6
Valeryl	3·5

DISCUSSION

Although kynurenine formamidase and the chick-kidney enzyme both catalyse removal of *N*-acyl groups from substituted acylanilides, there are clear differences between them. The main points of contrast are summarized in Table 8.

From the way in which they were separated it seems reasonable to conclude that most of the particles present in the suspensions used were mitochondria (Hogeboom, Schneider & Palade, 1948; Potter, Recknagel & Hurlbert, 1951; Schneider, 1959). Similarly, most of the microsomes were removed with the supernatant from the second centrifuging. Failure of a variety of treatments to release activity into the 15 000 *g* supernatant indicates that the enzyme is very tightly bound to the mitochondria. On the other hand, activity does not depend upon structural integrity of the mitochondria, for this could be severely damaged without great loss of activity. Deoxycholate and Triton X-100, for instance, caused a release of 60–70% of mitochondrial protein yet left nearly all of the original activity in a form still sedimentable at 15 000 *g*.

A high sensitivity to inhibition by *p*-chloromercuribenzoate, an even higher sensitivity to heavy-metal inhibition, and the partial reversal of the former by glutathione and cysteine all point towards the presence of thiol groups in the region of the enzyme's active site.

Because these experiments were done with relatively crude enzyme preparations (mitochondrial suspensions) it would be unwise to assume that a single enzyme is responsible for the hydrolysis of all the substrates that reacted; on the other hand the simplest hypothesis is to attribute observed activity to but a single enzyme. In this connexion it might be pointed out that the hydrolyses by mitochondrial-bound enzyme of two substrates so different as *p*-acetamidoanisole and *p*-benzamidobenzoic acid have the same pH–activity

Table 8. *Some points of difference between kynurenine formamidase of rat-liver and the chick-kidney deacylase*

	Kynurenine formamidase	Chick-kidney deacylase
Subcellular localization	Cell sap	Mitochondria
Sensitivity to inhibition by metals	Low	High
Optimum *N*-acyl group	Formyl	Acetyl or propionyl
Optimum ring substitution	*ortho*	*para*
Physiological substrate	Formylkynurenine	Unknown

relationships and the same sensitivities to inhibition by *p*-chloromercuribenzoate. Moreover, from the extremely low rate at which *o*-formamidobenzoic acid was hydrolysed, it can be concluded that the deacylation of *ortho*-substituted compounds was not due to the presence of small amounts of kynurenine formamidase. But not until activity has been released into solution and further fractionated can the question of the number of enzymes involved be satisfactorily examined.

For the same reason caution must be used in the interpretation of the kinetic data since diffusion to an active site, rather than formation of a Michaelis–Menten complex, could be a rate-limiting factor. In argument against this might be quoted the high apparent K_m value of formanilide (7·4 mM) as compared with the greater apparent affinities of larger molecules such as *p*-hexanamidobenzoic acid (K_m 0·54 mM) and *p*-benzamidobenzoic acid (K_m 1·3 mM), although this would ignore the possibility of a more rapid diffusion by more highly lipophilic compounds. Whatever the true situation might be, it seems most unlikely that rate-limitation imposed by diffusion can explain the more obvious differences encountered.

More rapid hydrolysis of chloroacetamido than of acetamido derivatives, seen with both chick-kidney and rat-liver enzymes, may be a fairly general phenomenon. It is well known with hydrolysis of *N*-acyl amino acids (Birnbaum, Levintow, Kingsley & Greenstein, 1952) and was noted by Gomori (1954) in hydrolysis of *N*-acyl naphthylamines by human tissues; it is consistent with the readier catalysis of hydrolysis by H^+ and OH^- ions. Yet formamido compounds, even more labile to acid and base hydrolysis, are hydrolysed by chick kidney at maximal rates no greater than those of the acetamido homologues.

Slow rates of hydrolysis of substrates with *ortho* substituents can probably not, at least in the two cases where apparent K_m was measured, be entirely explained by low affinities for the enzyme. Steric hindrance to hydrolysis offered by the *ortho* substituent is another important factor to be taken into account, and may explain the resistance of 1-acetamidonaphthalene to hydrolysis as compared with the 2-isomer. The effect of *ortho* substitution upon activity is in marked contrast with hydrolysis catalysed by kynurenine formamidase, which for optimum conditions demands an *ortho* substituent; it implies a fundamental difference not only between structural arrangements within the active centres but also between the mechanisms of catalysis of the two enzymes. By using a pair of substrates such as *o*-formamidobenzoic acid and *p*-acetamidobenzoic acid the amount of either or both enzymes within a particular tissue can be measured.

A physiological function, if any, for the chick-kidney enzyme is still to be found. A preliminary survey shows it to be absent from the kidney and liver of all mammalian species examined. It is present in varying amounts in the kidney of a variety of birds, particularly in the less-specialized orders, but in some species of the more recent natural orders it has almost disappeared. It has also been found in a high proportion of the reptile species so far examined. Possibly it is a vestigial enzyme surviving from a time when it played a part, no longer important, in the organism's economy. Although it is undesirable to speculate too freely, particularly as a natural substrate may be awaiting discovery, the enzyme may have been acquired at a stage in chordate evolution when some arylamine other than formylkynurenine was of biochemical importance but was in danger of being rendered metabolically inaccessible by increasing powers of acetylation. It is interesting that arylamines can act as acceptors in the liver acetylating system, that activity is higher in pigeon than in mammalian liver and that acceptors of this type include *p*-aminobenzoic acid (Lipmann, 1945; Tabor, Mehler & Stadtman, 1953). No chordate is known which can synthesize its own folic acid from *p*-aminobenzoic acid, but if chordates existed which could perform this synthesis, and if they possessed also a strong acetylating system which accidentally includes arylamines, then acquisition of a mechanism deacetylating the essential metabolite would have been an advantage. In this hypothetical situation loss of the ability to synthesize folic acid would make the deacetylating enzyme redundant and species which lost the enzyme through genetic mutation would be at no selective disadvantage.

SUMMARY

1. Homogenates of chick kidney rapidly deacylate a variety of acylanilides. Activity is associated with the mitochondria, from which it cannot be released by treatment with several surface-active agents or by repeated freezing and thawing. Some properties of the enzyme have been investigated in mitochondrial suspensions.

2. Deacylation is most rapid in phosphate buffer at pH 7·3 and at 44°. Heat of activation has been calculated as 15 300 cal.

3. Heavy metals inhibit the enzyme strongly. At concentrations as low as 0·2 μM, Hg^{2+} ion inhibits by about 20%. Inhibition by *p*-chloromercuribenzoate (50% at 50 μM) is partly reversed by reduced glutathione and cysteine.

4. Substitution in the *para* position of acetanilide generally results in an increase in the relative rate of deacetylation and a decrease in apparent K_m. *Ortho* substitution has a marked effect in the

opposite direction; the decrease in rate (which may be 1000-fold) at moderate substrate concentration can be accounted for only partly by decreased affinity of substrate for enzyme.

5. The effect of increasing the *N*-acyl chain length from formyl to hexanoyl has been tested. Hydrolysis is fastest at acetyl or propionyl, depending upon the nature of the *para* substituent. Of all the substrates tested *p*-acetamidophenylacetic acid has the lowest apparent K_m (78 μM). Chloroacetylated compounds are hydrolysed most rapidly; *p*-chloroacetamidoanisole, for instance, is deacylated at a maximal rate corresponding to 6·1 m-moles/g. of fresh tissue/hr. Some *N*-benzoyl derivatives of *p*-aminobenzoic acid are slowly hydrolysed.

6. On the basis particularly of distribution, subcellular localization, sensitivity to metal inhibition and the effect on rate of ring substitution and *N*-acyl chain length the enzyme is seen to have properties very different from those of kynurenine formamidase.

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Purification of a Glycoprotein from Bovine-Submaxillary Glands

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Investigations of the composition of purified mucins from salivary glands have been made by Tanabe (1939), Blix, Svennerholm & Werner (1952) and Blix, Lindberg, Odin & Werner (1956). Degradative procedures were employed in the preparations, since the purpose was to study the composition of the carbohydrate or of the sialic acid. A purified material was prepared by Heimer & Meyer (1956) from mucin clots obtained from extracts of bovine-submaxillary glands. A 6M-urea solution adjusted to pH 9·0 was used for the extraction of acetone-extracted material.

In earlier work from this Laboratory (Nisizawa & Pigman, 1959), mucin clots from bovine-submaxillary glands were freeze-dried as sodium salts. The materials retained the viscosity of the extracts and were soluble in water. The procedure was a modification of the method of Hammarsten (1888). These clots, composed of an anionic glycoprotein combined with a protein cation, precipitated between pH 3·5 and 4·0.

The present work deals with the purification and further investigation of the physical characteristics and chemical composition of the material mainly responsible for the viscous nature of the mucin clot. Two preparations obtained at intermediate stages of purification have also been analysed.

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EXPERIMENTAL

Source material. In the previous work (Nisizawa & Pigman, 1959) the sodium salt of the bovine-submaxillary mucin clot was obtained as follows. Bovine-submaxillary